APPENDIX A. PROCEDURES FOR MAKING SOLUTIONS, CULTURE MEDIA, PLATE AGAR MEDIA, CSC OR POSITIVE CONTROL STOCK SOLUTIONS AND WORKING DILUTIONS, ETC.

1. Ampicillin Solutions:

A. For Plate Incorporation (Culture Reisolation):

1. Make up a **14.53 mg/ml ampicillin trihydrate solution:**
   a. Weigh out 0.07265 g of ampicillin trihydrate (FW = 403.47; anhydrous FW = 349.4).
   b. Dilute to 5 ml volume with sterile 0.02 N NaOH.
   c. Mix well to dissolve. Store frozen at -20°C.
2. Add 2 ml of the 14.53 mg/ml ampicillin trihydrate solution to one liter of ~45°C sterile agar [either Vogel Bonner minimal glucose agar media (solution 12.B) or Oxoid nutrient agar media (solution 9.B)] to give a final concentration of ~ **25 ug/ml** of ampicillin in the agar media. Dispense 30 ml of the agar media per plate.

B. For incorporation in Oxoid nutrient broth culture media (Culture Reisolation):

1. Make up a **1.479 mg/ml ampicillin trihydrate solution:**
   a. Weigh out 0.01479 g of ampicillin trihydrate (FW = 403.468; anhydrous FW = 349.4).
   b. Dilute to 10 ml volume with sterile 0.02 N NaOH.
   c. Mix well to dissolve.
2. Add 2 ml of the 1.479 mg/ml ampicillin solution to 100 ml of sterile Oxoid nutrient broth (solution 8.B) at room temperature to give a final concentration of ~ **25 ug/ml** ampicillin in the broth media.

2. **Biotin Solutions:**

A. For soft (top) agar supplement to test for the histidine requirement:

1. Make up a **0.5 mM biotin** solution:
   a. Weigh out 0.12215 g of D-biotin (FW = 244.3) into one liter of deionized distilled water.
   b. Mix well to dissolve.
   c. Filter sterilize through a 0.2 u Nalgene filter unit and store refrigerated.
   d. Add 10 ml to 100 ml of soft (top) agar.
   e. Use 2.5 ml of this biotin supplemented soft agar (solution 10) to plate 0.1 ml of a test culture. Incubate the plate overnight at 37°C. There should be no bacterial growth evident since there is a histidine requirement for the normal growth of these strains.

B. For Routine Soft (Top) Agar Supplementation:

1. Make up a **0.5 mM biotin + 0.5 mM L-histidine HCl solution:**
   a. Weigh out D-biotin and L-histidine HCl and QS to the appropriate volume with deionized distilled water:

	g/100 ml	g/200 ml	g/500 ml	g/liter
L-histidine HCl (FW = 191.7)	0.009585 g	0.01917 g	0.047925 g	0.09585 g
D-biotin (FW = 244.3)	0.012215 g	0.02443 g	0.061075 g	0.12215 g

   b. Mix well to dissolve. At room temperature, it may take all day to dissolve. Recent recommendations to heat the water to the boiling point have been made by Maron and Ames **[14]**.
   c. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

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2. Add 10 ml of this solution to each 100 ml of sterile soft agar (solution 10). Use 2.5 ml of this supplemented soft

(top) agar solution for plating the test compound, the culture and the S9 mix during routine testing.

C. OPTIONAL METHOD--For Soft (Agar) Supplement used to Plate Viable Counts (Titers) on Vogel Bonner Minimal Glucose Agar Plates:

1. Make up **0.5 mM biotin + 0.66 ug L-histidine HCl/ml** solution:

a. Weigh out D-biotin and L-histidine HCl and QS to the appropriate volume with deionized distilled water:

	g/100 ml	g/200 ml	g/500 ml	g/liter
L-histidine HCl (FW = 191.7)	0.06600 g	0.13200 g	0.33000 g	0.6600 g
D-biotin (FW = 244.3)	0.012215 g	0.02443 g	0.061075 g	0.12215 g

b. Mix well to dissolve.

c. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

2. Add 10 ml of this solution to each 100 ml of sterile soft agar. Use 2.5 ml of this supplemented soft (top) agar solution for plating titer dilutions of each overnight (16 hour) culture on Vogel Bonner minimal glucose agar plates. [Note: This is an alternative or optional procedure for plating titers. Normally, 0.1 ml of a 50.42 ug/ml histidine solution (solution 3) is pipetted into a tube containing 0.1 ml of the titer dilution which is then plated using the routine soft agar (solution 10) supplemented with histidine and biotin (see solution 2.B.).]

3. Histidine Solutions:

A. Super His Solution - For Plating Viable Counts (Titers) on Vogel Bonner Minimal Glucose Agar Plates in conjunction with the Routine Soft (Top) Agar:

1. Make up a **50.42 ug/ml histidine HCl** solution:

a. Weigh out 0.05042 g of L-histidine HCl.

b. QS to 100 ml with deionized distilled water.

c. Mix well to dissolve.

d. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

2. Add 0.1 ml to each sterile culture tube to be used for plating viable counts (titers), crystal violet sensitivity, ampicillin resistance and colony morphology. Next, add 0.1 ml of the appropriate titer dilution. Using 2.5 ml of the soft agar (supplemented with solution 2.B.), plate mixture on Vogel Bonner minimal glucose agar plates.

4. Crystal Violet Solution (1 mg/ml):

A. Weigh out 10.6 mg of crystal violet (94% purity).

B. QS to 10 ml volume with sterile deionized distilled water.

C. Mix well to dissolve.

D. To check the rfa (deep rough mutation) marker:

1. Add 10 ul of the 1 mg/ml crystal violet solution to a sterile 6.35 mm (1/4") diameter BBL blank sensi disc and then place on Vogel Bonner minimal glucose agar plate to which biotin and excess histidine have been added with 0.1 ml of the overnight (16 hour) cell culture.

2. Incubate at 37°C.

3. A zone of ~14-18 mm inhibition around each disc indicates that the rfa marker is intact.

5. Glucose Solution (20 g/100 ml) for Vogel Bonner Minimal Glucose Agar Plates:

A. Weigh out 200 g of dextrose.

B. QS to one liter volume with deionized distilled water.

C. Mix well to dissolve all of the dextrose (low heat may have to be used).

D. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

E. Using disposable sterile 100 ml graduated cylinders (or sterile 100 ml glass cylinder), add 100 ml of this glucose solution to 400

ml of sterile 2X Vogel Bonner salts. This solution may be stored at room temperature under sterile conditions.

6. 0.9% Sodium Chloride (NaCl) Solution (Saline solution for making titer dilutions):

A. Weigh out 9 g NaCl.

B. QS to one liter volume with deionized distilled water.

C. Mix well to dissolve.

D. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

E. Use 4.5 ml of this sterile saline solution for making titer dilutions.

7. 0.02 N Sodium Hydroxide (NaOH) Solution (for making ampicillin solutions):

A. Weigh out 0.8 g NaOH and dilute to one liter volume with deionized distilled water.

B. Filter sterilize through 0.2 u Nalgene filter units and store refrigerated.

C. Use to make up ampicillin solutions (See solutions 1.A. and 1.B.).

8. Oxoid Nutrient Broth Culture Media:

A. Oxoid nutrient broth fortified with 10 ug/ml L-histidine HCl:

1. Weigh out Oxoid Nutrient Broth No. 2 and L-histidine HCl:

	per 100 ml	per 500 ml	per 1 L	per 2 L
Oxoid nutrient broth No. 2	2.5 g	12.5 g	25 g	50 g
L-histidine HCl	1 mg	5 mg	10 mg	20 mg

2. QS to the appropriate volume with deionized distilled water.

3. Mix well to dissolve.

4. Divide this mixture into 100 ml aliquots in 250 ml screw capped Erlenmeyer flasks.

5. Autoclave at 250° F for 30 minutes and store at room temperature.

6. Use to dispense the appropriate volumes for overnight (16 hour) cultures, dilutions of overnight cultures or for blanks used to measure the optical density of overnight (16 hour) cultures on a Klett-Summerson photoelectric colorimeter or the Hitachi spectrophotometer.

B. Oxoid nutrient broth fortified with 10 ug/ml of L-histidine HCl and 25 ug/ml ampicillin (Culture reisolation):

1. Weigh out Oxoid nutrient broth No. 2 and L-histidine HCl (see chart for solution 8.A. above).
2. QS to the appropriate volume with deionized distilled water.
3. Mix well to dissolve.
4. Divide into 100 ml aliquots in 250 ml screw cap Erlenmeyer flasks.
5. Autoclave at 250° F for 30 minutes and cool media to room temperature.
6. Add 2 ml of a 1.479 mg/ml ampicillin trihydrate solution (see solution 1.B.) to 100 ml of culture media to give a final concentration of ~ 25 ug/ml of ampicillin.
7. Store a room temperature.
8. Use for growing up overnight cultures under ampicillin pressure.

9. Oxoid Nutrient Agar Plates:

A. Oxoid nutrient agar plates fortified with 10 ug/ml of L-histidine HCl:

1. Weigh out 25 g Oxoid nutrient broth No. 2, 10 mg L-histidine HCl, and 15 g Difco Bacto-agar into one liter of deionized distilled water (use 2 L Erlenmeyer flasks).
2. Autoclave for 45 minutes at 250 ° F.
3. Dispense 30 ml of the sterile nutrient agar media per plate using a New Brunswick Scientific dispenser pump and sterile tubing. [**Note**: Work performed in a laminar flow hood will minimize contamination of the plates. Also, plates should be inverted after they have hardened to prevent excess droplets

of moisture from forming directly on the surface of the agar. Plates may be store refrigerated for several weeks.]

B. Oxoid nutrient agar plates fortified with 10 ug/ml of L-histidine HCl and 25 ug/ml of ampicillin (Culture reisolation):

1. Weigh out 25 g Oxoid nutrient broth No. 2, 10 mg L-histidine HCl and 15 g Difco Bacto-agar into one liter of deionized distilled water.
2. Autoclave for 45 minutes at 250° F.
3. Cool to approximately 45° C in a water bath.
4. Add 2 ml of a 14.53 mg/ml ampicillin trihydrate solution (see solution 1.A.) to give approximately 25 ug of ampicillin per ml of agar media.
5. Dispense 30 ml of the sterile agar media per plate using a New Brunswick Scientific dispenser pump. **[See previous note.]**

10. Soft (Top) Agar for Overlays:

A. Weigh out Difco Bacto-agar and NaCl into Erlenmeyer flasks:

	per 100 ml	per 500 ml	per 1 L
Difco Bacto-agar	0.6 g	3.0 g	6.0 g
NaCl	0.5 g	2.5 g	5.0 g
deionized distilled water	100 ml	500 ml	1000 ml
--after autoclaving			
0.5 mM histidine + 0.5 mM biotin (see 2.B.)	10 ml	50 ml	100 ml
Autoclave time	30 minutes	40 minutes	45 minutes

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B. Add the appropriate volume of deionized distilled water.

C. Autoclave for the appropriate time at 250°F.

D. Cool media to ~ 45°C in a heated water bath.

E. Add 10 ml of the appropriate soft agar supplement (histidine-biotin solutions) per 100 ml of soft agar before dispensing 2.5 ml of the supplemented soft agar into the culture tubes used for plating.

11. Vogel Bonner 50X salt solution for making Vogel Bonner Minimal Glucose agar plates [Reference 43]:

A. Weigh out the components for this solution separately:

	per 1 L	per 2 L	per 3 L	per 4 L	per 16 L
ml deionized distilled water	670 ml	1340 ml	2010 ml	2680 ml	10,720 ml
g magnesium sulfate, 7-hydrate	10 g	20 g	30 g	40 g	160 g
g citric acid, monohydrate	100 g	200 g	300 g	400 g	1600 g
g potassium phosphate, dibasic, anhydrous	500 g	1000 g	1500 g	2000 g	8000 g
g sodium ammonium phosphate, 4-hydrate	175 g	350 g	525 g	700 g	2800 g

B. Mix each compound completely in the order indicated with the specified volume of deionized distilled water in order to avoid precipitation. Adjust final volume if necessary with deionized distilled water.

C. Store at room temperature in a Nalgene dispenser (Note: a few ml of chloroform may be added as a preservative).

12. Vogel Bonner Minimal Glucose Agar:

A. For Test Plates:

1. Weigh out 15 g of Difco Bacto-agar into a one liter Erlenmeyer flask; add 500 ml deionized distilled water; autoclave for 40 minutes at 250° F.
2. Measure 20 ml of the Vogel Bonner 50 X salt solution (see solution 11) into a one liter Erlenmeyer flask; add 380 ml deionized distilled water; autoclave for 40 minutes at 250°F. Note: This component solution can be made up, sterilized and stored at room temperature ahead of time.
3. Add 100 ml sterile glucose solution to the salt portion (2X sterile Vogel Bonner salts).

4. Pour the salt/glucose solution into the agar solution.
5. Dispense 30 ml of the sterile agar media plus glucose per plate using a New Brunswick Scientific dispenser pump and sterile tubing. [**Note**: Work performed in a laminar flow hood will minimize contaminants on the plates. Plates should be inverted after they have hardened in order to prevent the formation of moisture droplets on the surface of the agar. The plates may be stored refrigerated for several weeks.]

B. For Vogel Bonner plates that could be used to plate viable counts to test for ampicillin resistance in strains TA98 and TA100:

1. Prepare Vogel Bonner minimal glucose agar media as above (see solution 12.A.).
2. Before dispensing, add 2 ml of a 14.53 mg/ml ampicillin trihydrate solution (see solution 1.A.) per liter of sterile agar to give a final concentration of ~ 25 ug/ml of ampicillin in the agar media.
3. Dispense 30 ml of the sterile agar/glucose/ampicillin media per plate using a New Brunswick Scientific dispenser pump and sterile tubing. **[See previous note above.]**

13. Sodium Phosphate Buffer:

A. Stock Solutions: Make up stock solutions according to the following table. Solution A is the sodium phosphate, monobasic solution. Solution B is the sodium phosphate, dibasic solution.

Stock Solution	Compound	g/500 ml	g/1 liter
0.2 M NaH_2PO_4 (soln A)	$NaH_2PO_4(H_2O)$	13.6 g	27.2 g
0.2 M Na_2HPO_4 (soln B)	$Na_2HPO_4(7H_2O)$	26.83 g	53.66 g

1. Mix up these two stock solutions separately.
2. QS to the appropriate volume with deionized distilled water.

B. Sodium Phosphate Buffer, pH = 7.4: Make up buffer according to the following table.

Stock Solution	ml/100 ml	ml/500 ml	ml/1 liter	ml/2 liter
(A) 0.2 M NaH_2PO_4	19 ml	95 ml	190 ml	380 ml
(B) 0.2 M Na_2HPO_4	81 ml	405 ml	810 ml	1620 ml
Autoclave time	30 minutes	40 minutes	45 minutes	55 minutes

1. Check the pH of the final solution and adjust to pH 7.4 if necessary with the stock solutions [the NaH_2PO_4 solution (solution A) will lower the pH; the Na_2HPO_4 solution (solution B) will raise the pH].
2. Autoclave at 250 °F for the appropriate time.

14. Ames Salt Solution for the S9 Mix (0.4 M $MgCl_2$ + 1.65 M KCl):

A. Make up the salt solution according to the following table:

Compound	g/100 ml	g/ 500 ml	g/1 liter
$MgCl_2$	8.13 g	40.65 g	81.3 g
KCl	12.3 g	61.5 g	123.3 g
Autoclave time	30 min.	40 min.	45 min.

B. QS to the appropriate volume with deionized distilled water.

C. Mix well to dissolve.

D. Autoclave at 250°F for the appropriate time.

15. -S9 Mix:

A. Make up the -S9 solution according to the following table:

Solution	per 1 ml	per 4 L
Ames salt solution (solution 14)	0.02 ml	80 ml
Sodium phosphate buffer (" 13.B)	0.50 ml	2000 ml
deionized distilled water	0.41 ml	1640 ml

Note: the sodium phosphate buffer and Ames salt solutions must be completely cooled before mixing, otherwise, a precipitate will occur.

B. Mix solutions and water thoroughly.

C. Filter sterilize through 0.2 u Nalgene filter units (Note: Do not autoclave this final solution since a precipitate will form) and store refrigerated.

16. + S9 Mix Solutions:

A. FOR STANDARD PLATE INCORPORATION ASSAY - + S9 Mix [8 mM Magnesium chloride; 33 mM potassium chloride; 5 mM glucose-6-phosphate; 4 mM NADP (TPN); 100 mM sodium phosphate buffer, pH 7.4; 0.070 ml of S9/ml mix (35 ul of S9 per plate)]:

Prepare the +S9 mix solution fresh the day of the experiment and keep on ice according to the experimental volume needed on the following chart [Note: the appropriate amount of the -S9 mix (solution 15 above) can be used for this recipe instead of the individual salt, buffer and water solutions.]:

Volume ======>	1 ml	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml
ml -S9 Mix	0.93	4.65	9.3	13.95	18.6	23.25	27.9
ml S9	0.07	0.35	0.7	1.05	1.4	1.75	2.2
g NADP	0.00343	0.01717	0.03434	0.05152	0.06869	0.08586	0.10303
g G-6-P	0.00146	0.00730	0.01460	0.02190	0.02920	0.03650	0.04380

[+S9 Mix for Standard Plate Incorporation Assay (35 ul S9/plate) continued]

Volume ======>	35 ml	40 ml	45 ml	50 ml	55 ml	60 ml	65 ml
ml -S9 Mix	32.55	37.2	41.85	46.5	51.15	55.8	60.45
ml S9	2.45	2.8	3.15	3.5	3.85	4.2	4.55
g NADP	0.12020	0.13738	0.15455	0.17172	0.18889	0.20606	0.22324
g G-6-P	0.05110	0.05840	0.06570	0.07300	0.08030	0.08760	0.09490

Volume ======>	70 ml	75 ml	80 ml	85 ml	90 ml	95 ml	100 ml
ml -S9 Mix	65.1	69.75	74.4	79.05	83.7	88.35	93.0
ml S9	4.9	5.25	5.6	5.95	6.3	6.65	7.0
g NADP	0.24041	0.25758	0.27475	0.29192	0.30910	0.32627	0.34344
g G-6-P	0.10220	0.10959	0.11680	0.12410	0.13140	0.13870	0.14600

Volume ======>	105 ml	110 ml	115 ml	120 ml	125 ml	130 ml	135 ml
ml -S9 Mix	97.65	102.3	106.95	111.6	116.25	120.9	125.55
ml S9	7.35	7.7	8.05	8.4	8.75	9.1	9.45
g NADP	0.36061	0.37778	0.39496	0.41213	0.42930	0.44647	0.46364
g G-6-P	0.15330	0.16060	0.16790	0.17520	0.18250	0.18980	0.19710

Volume ======>	140 ml	145 ml	150 ml	155 ml	160 ml	165 ml	170 ml
ml -S9 Mix	130.2	134.85	139.5	144.15	148.8	153.45	158.1
ml S9	9.8	10.15	10.5	10.85	11.2	11.55	11.9
g NADP	0.48082	0.49799	0.51516	0.53233	0.54950	0.56668	0.58385
g G-6-P	0.20440	0.21170	0.21900	0.22630	0.23360	0.24090	0.24820

Volume ======>	175 ml	180 ml	185 ml	190 ml	195 ml	200 ml	205 ml
ml -S9 Mix	162.75	167.4	172.05	176.7	181.35	186.0	190.65
ml S9	12.25	12.6	12.95	13.3	13.65	14.0	14.35
g NADP	0.60102	0.61819	0.63536	0.65254	0.66971	0.68688	0.70405
g G-6-P	0.25550	0.26280	0.27010	0.27740	0.28470	0.29200	0.29930

Volume ======>	210 ml	215 ml	220 ml	225 ml	230 ml	235 ml	240 ml
ml -S9 Mix	195.3	199.95	204.6	209.25	213.9	218.55	223.2
ml S9	14.7	15.05	15.4	15.75	16.1	16.45	16.8
g NADP	0.72122	0.73840	0.75557	0.77274	0.78991	0.80708	0.82426
g G-6-P	0.30660	0.31390	0.32120	0.32850	0.33580	0.34310	0.35040

[+S9 Mix for Standard Plate Incorporation Assay (35 ul S9/plate) continued]

Volume ======>	245 ml	250 ml	255 ml	260 ml	265 ml	270 ml	275 ml
ml -S9 Mix	227.85	232.5	237.15	241.8	246.45	251.1	255.75
ml S9	17.15	17.5	17.85	18.2	18.55	18.9	19.25
g NADP	0.84143	0.85860	0.87577	0.89294	0.91012	0.92729	0.94446
g G-6-P	0.35770	0.36500	0.37230	0.37960	0.38690	0.39420	0.40150

Volume ======>	280 ml	285 ml	290 ml	295 ml	300 ml	305 ml	310 ml
ml -S9 Mix	260.4	265.05	269.7	274.35	279.0	283.65	288.3
ml S9	19.6	19.95	20.3	20.65	21.0	21.35	21.7
g NADP	0.96163	0.97880	0.99598	1.01315	1.03032	1.04749	1.06466
g G-6-P	0.40880	0.41610	0.42340	0.43070	0.43800	0.44530	0.45260

Volume ======>	315 ml	320 ml	325 ml	330 ml	335 ml	340 ml	345 ml
ml -S9 Mix	292.95	297.6	302.25	306.9	311.55	316.2	320.85
ml S9	22.05	22.4	22.75	23.1	23.45	23.8	24.15
g NADP	1.09184	1.09901	1.11618	1.13335	1.15052	1.16770	1.18487
g G-6-P	0.45990	0.46720	0.47450	0.48180	0.48910	0.49640	0.50370

B. FOR S9 COMPARISONS ONLY - + S9 Mix [8 mM Magnesium chloride; 33 mM potassium chloride; 5 mM glucose-6-phosphate; 4 mM NADP (TPN); 100 mM sodium phosphate buffer, pH 7.4; 0.090 ml of S9/ml mix (45 ul of S9 per plate)]:

Prepare the +S9 mix solution fresh the day of the experiment and keep refrigerated or on ice according to the experimental volume needed on the following chart:

Volume ======>	1 ml	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml
ml -S9 Mix	0.91	4.55	9.1	13.65	18.2	22.75	27.3
ml S9	0.09	0.45	0.9	1.35	1.8	2.25	2.7
g NADP	0.00343	0.01717	0.03434	0.05152	0.06869	0.08586	0.10303
g G-6-P	0.00146	0.00730	0.01460	0.02190	0.02920	0.03650	0.04380

Volume ======>	35 ml	40 ml	45 ml	50 ml	55 ml	60 ml	65 ml
ml -S9 Mix	31.85	36.4	40.95	45.5	50.05	54.6	59.15
ml S9	3.15	3.6	4.05	4.5	4.95	5.4	5.85
g NADP	0.12020	0.13738	0.15455	0.17172	0.18889	0.20606	0.22324
g G-6-P	0.05110	0.05840	0.06570	0.07300	0.08030	0.08760	0.09490

Volume ======>	70 ml	75 ml	80 ml	85 ml	90 ml	95 ml	100 ml
ml -S9 Mix	63.7	68.25	72.8	77.35	81.9	86.45	91.0
ml S9	6.3	6.75	7.2	7.65	8.1	8.55	9.0
g NADP	0.24041	0.25758	0.27475	0.29192	0.30910	0.32627	0.34344
g G-6-P	0.10220	0.10959	0.11680	0.12410	0.13140	0.13870	0.14600

Volume ======>	105 ml	110 ml	115 ml	120 ml	125 ml	130 ml	135 ml
ml -S9 Mix	95.55	100.1	104.65	109.2	113.75	118.3	122.85
ml S9	9.45	9.9	10.35	10.8	11.25	11.7	12.15
g NADP	0.36061	0.37778	0.39496	0.41213	0.42930	0.44647	0.46364
g G-6-P	0.15330	0.16060	0.16790	0.17520	0.18250	0.18980	0.19710

[+S9 Mix for S9 comparisons (45 ul S9/plate) continued]

Volume ======>	140 ml	145 ml	150 ml	155 ml	160 ml	165 ml	170 ml
ml -S9 Mix	127.4	131.95	136.5	141.05	145.6	150.15	154.7
ml S9	12.6	13.05	13.5	13.95	14.4	14.85	15.3
g NADP	0.48082	0.49799	0.51516	0.53233	0.54950	0.56668	0.58385
g G-6-P	0.20440	0.21170	0.21900	0.22630	0.23360	0.24090	0.24820

Volume ======>	175 ml	180 ml	185 ml	190 ml	195 ml	200 ml	205 ml
ml -S9 Mix	159.25	163.8	168.35	172.9	177.45	182.0	186.55
ml S9	15.75	16.2	16.65	17.1	17.55	18.0	18.45
g NADP	0.60102	0.61819	0.63536	0.65254	0.66971	0.68688	0.70405
g G-6-P	0.25550	0.26280	0.27010	0.27740	0.28470	0.29200	0.29930

Volume ======>	210 ml	215 ml	220 ml	225 ml	230 ml	235 ml	240 ml
ml -S9 Mix	191.1	195.65	200.2	204.75	209.3	213.85	218.4
ml S9	18.9	19.35	19.8	20.25	20.7	21.15	21.6
g NADP	0.72122	0.73840	0.75557	0.77274	0.78991	0.80708	0.82426
g G-6-P	0.30660	0.31390	0.32120	0.32850	0.33580	0.34310	0.35040

Volume ======>	245 ml	250 ml	255 ml	260 ml	265 ml	270 ml	275 ml
ml -S9 Mix	222.95	227.5	232.05	236.6	241.15	245.7	250.25
ml S9	22.05	22.5	22.95	23.4	23.85	24.3	24.75
g NADP	0.84143	0.85860	0.87577	0.89294	0.91012	0.92729	0.94446
g G-6-P	0.35770	0.36500	0.37230	0.37960	0.38690	0.39420	0.40150

Volume ======>	280 ml	285 ml	290 ml	295 ml	300 ml	305 ml	310 ml
ml -S9 Mix	254.8	259.35	263.9	268.45	273.0	277.55	282.1
ml S9	25.2	25.65	26.1	26.55	27.0	27.45	27.9
g NADP	0.96163	0.97880	0.99598	1.01315	1.03032	1.04749	1.06466
g G-6-P	0.40880	0.41610	0.42340	0.43070	0.43800	0.44530	0.45260

Volume ======>	315 ml	320 ml	325 ml	330 ml	335 ml	340 ml	345 ml
ml -S9 Mix	286.65	291.2	295.75	300.3	304.85	309.4	313.95
ml S9	28.35	28.8	29.25	29.7	30.15	30.6	31.05
g NADP	1.09184	1.09901	1.11618	1.13335	1.15052	1.16770	1.18487
g G-6-P	0.45990	0.46720	0.47450	0.48180	0.48910	0.49640	0.50370

17. **Positive Controls**

Note: Positive control stock solutions are prepared in a glove box. Refer to reference 51 for the procedures. Test solution are prepared in a chemical fume hood.

A. 2AA (2-aminoanthracene):

1. Stock Solution:

a. Weigh out 40 mg of 2AA and dissolve in 20 ml DMSO to give a stock concentration of 2 mg/ml.

b. Dispense 0.1 ml aliquots of the 2 mg/ml 2AA solution into capped cryule tubes.

c. Store at -20°C. This makes approximately 200 tubes of the 2AA stock (200 ug/0.1 ml).

2. Testing Solution (0.5 ug/plate):

a. Add 1.9 ml DMSO to the 0.1 ml of thawed 2AA stock to make a 100 ug/ml 2AA solution.

b. Take 0.1 ml of the 100 ug/ml 2AA solution and dilute with 1.9 ml DMSO to make a 5 ug/ml 2AA solution.

c. As a positive control for strains TA98 and TA100 with metabolic activation, plate 0.1 ml of the 5 ug/ml 2AA solution in the standard assay (0.5 ug of 2AA/plate).

B. 2NF (2-nitrofluorene):

1. Stock Solution:

a. Weigh out 10 mg of 2NF and dissolve in 20 ml DMSO to give a stock concentration of 0.5 mg/ml.

b. Dispense 0.1 ml aliquots of the 0.5 mg/ml 2NF solution into capped cryule tubes.

c. Store at -20°C. This makes approximately 200 tubes of the 2NF stock (50 ug/0.1 ml).

2. Testing Solution (2.5 ug/plate):

a. Add 1.9 ml DMSO to the 0.1 ml of thawed 2NF stock to make a 25 ug/ml 2NF solution.

b. As a positive control for strain TA98 without metabolic activation, plate 0.1 ml of the 25 ug/ml 2NF solution in the standard assay (2.5 ug of 2NF/plate).

C. Sodium Azide (NaN_3) :

1. Stock Solution:

a. Weigh out 40 mg of sodium azide and dissolve in 20 ml sterile deionized distilled water to give a stock concentration of 2 mg/ml.

b. Dispense 0.1 ml aliquots of the 2 mg/ml sodium azide solution into capped cryule tubes.

c. Store at room temperature. This makes approximately 200 tubes of the sodium azide stock (200 ug/0.1 ml).

2. Testing Solution (0.5 ug/plate):

a. Add 1.9 ml deionized distilled water to the 0.1 ml of sodium azide stock (stored at room temperature) to make a 100 ug/ml sodium azide solution.

b. Take 0.1 ml of the 100 ug/ml sodium azide solution and dilute with 1.9 ml deionized distilled water to make a 5 ug/ml sodium azide solution.

c. As a positive control for strain TA100 without metabolic activation, plate 0.1 ml of the 5 ug/ml sodium azide solution in the standard assay (0.5 ug of sodium azide per plate). The solvent control in this case is deionized distilled water.

18. Impaction trapped (IT) Cigarette Smoke Condensate (CSC) Solutions:

A. Preparation of IT CSC: See Reference 13 for IT CSC collection.

B. IT CSC Stock Solutions (10 mg/ml):

1. The weight of the CSC is provided by Project 6908 personnel.

2. Add DMSO directly to CSC in the impaction trap flask to give a stock concentration of 10 mg of CSC/ml in DMSO. Mix well to fully dissolve the CSC in the DMSO. These operations are routinely performed by Project 6908 personnel.

C. IT CSC Dilutions for Testing in the S/M assay: Routinely 0.1 ml of IT CSC dilutions are tested at concentrations of 0.5, 0.10, 0.15 and 0.20 mg of CSC per plate. These concentrations are normally in the linear range of the dose response curve. The following table gives the information for making these dilutions:

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[CSC]/0.1 ml/plate	ml of 10 mg/ml stock	ml DMSO
0.05 mg	0.050	0.950
0.10 mg	0.100	0.900
0.15 mg	0.150	0.850
0.20 mg	0.200	0.800

19. Test Compound (Potential Tobacco Ingredient) Solutions:

A. Obtain sample and obtain information on the concentration, solubility, solvent, etc.

B. Prepare dilutions of the sample in either DMSO or another appropriate solvent to give a log scale dose range of 0.0001, 0.001, 0.01, 0.1, 1.0, and 10.0 mg/plate. The actual dose scheme used may depend on the amount of the sample that is available for testing in the S/M assay. Normally, serial dilutions are made from a 100 mg/ml stock solution of each sample. The solvent control for each sample will depend on the solvent used.

20. UV Sensitivity Check: The purpose of this check is to confirm the uvrB mutation.

A. With a sterile wire loop, streak the overnight (16 hour) cultures on Oxoid nutrient agar plates.

B. Cover half of the petri plate with foil or other suitable material.

C. Irradiate the plate with germicidal shortwave (254 nm) uv light at a distance of 33 cm for 8 seconds.

D. Incubate at 37°C for 12-24 hours. The side of the petri plate exposed to the uv light should not show any viability.